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Highlights

- DNA pools containing several ITDs could serve analytical validation of FLT3-ITD NGS assays
- Higher NGS read counts per sample provided higher number of FLT3-ITD variants
- The sensitivity of the FLT3-ITD NGS assay was 0.007% FLT3-ITD/total allele rate
- Below 0.1% ITD rate the reproducibility of the NGS quantitation was poor (CV%>25%)

A DNA pool of FLT3-ITD positive DNA samples can be used efficiently for analytical evaluation of NGS-based FLT3-ITD quantitation - testing several different ITD sequences and rates, simultaneously

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Abstract

Internal tandem duplication (ITD) in the fms-like tyrosine kinase 3 (*FLT3*) gene is one of the most frequent genetic alteration in acute myeloid leukemia (AML), and it is associated with worse clinical outcome. Not only the presence but also the size, localization and the rate of this variant or the presence of multiple ITDs has prognostic information. The traditional PCR based diagnostic methods cannot provide information about all of these parameters in one assay, however the application of next generation sequencing (NGS) technique can be a reliable solution for this diagnostic problem. In order to evaluate the analytical properties of an NGS-based FLT3-ITD detection assay a QC sample was prepared from DNA of AML patients containing 19 different FLT3-ITD variants identified by NGS. The higher the total read count was in a certain sample of the NGS run, the more ITD variant types could be detected. The maximal sensitivity of FLT3-ITD detection by NGS technique was as low as 0.007% FLT3-ITD/total allele rate, however, below 0.1% rate, the reproducibility of the quantitation was poor (CV>25%). DNA pools with several FLT3-ITDs can be used efficiently for analytical evaluation of NGS-based FLT3-ITD quantitation testing several different ITD sequences and rates, simultaneously.

Keywords: Fms-like tyrosine kinase 3 (FLT3); internal tandem duplication (ITD); deep next generation sequencing (NGS); analytical validation

List of Abbreviations: AML, acute myeloid leukemia; bp, base pair; CE, capillary electrophoresis FLT3, Fms-like tyrosine kinase 3; ITD, internal tandem duplication; NGS, next generation sequencing; TD, tandem duplication

1. Introduction

Internal tandem duplication (ITD) in the juxtamembrane/tyrosinkinase domain 1 region of the fms-like tyrosine kinase 3 (*FLT3*) gene is one of the most frequent genetic alteration associated with acute myeloid leukemia (AML) (Kappelmayer et al., 2007). It is a long-known fact that the presence of this variant is associated with worse clinical outcome in these patients. Data are somewhat contradictory, however, several lines of evidence suggest that not only the presence, but the rate of this variant compared to the amount of the wild type allele in the patients' DNA sample has prognostic information. Furthermore, the size of ITD and its location in the region of exon 14 - intron 14 - exon 15 or the presence of multiple ITDs are important factors as well (Buban et al., 2011; Kappelmayer et al., 2007; Schranz et al., 2018). Another application of FLT3-ITD determination can be minimal residual disease monitoring in AML that requires a quantitative, very sensitive assay with high reproducibility (Bibault et al., 2015; Levis et al., 2018; Schranz et al., 2018).

The traditional PCR based diagnostic methods cannot provide information about all of these parameters in one assay, as described in Table 1 (Abdelhamid et al., 2012; Alikian et al., 2017; Buban et al., 2011; Grunwald et al., 2014; Schiller et al., 2012; Takamatsu, 2017). Several studies have suggested that the application of next generation sequencing (NGS) technique can be a reliable solution for this diagnostic problem. Deep sequencing of the *FLT3* gene - after amplification - or captured DNA fragments of the patients' genome can identify different ITD sequences at the same time, providing also the size and the localization of these duplications. Furthermore, the rates of ITDs can be determined based on the number of the analyzed reads amplified from the ITD and wild type variants (Bibault et al., 2015; Levis et al., 2018; Schranz et al., 2018). An increase in the number of the total read count can enhance

the sensitivity of the NGS-based ITD assay, dramatically (Bibault et al., 2015; Levis et al., 2018).

Since the NGS FLT3-ITD assays provide quantitative data about the FLT3-ITD rate a proper analytical evaluation of these methods is required according to the recommendations of the American College of Molecular Genetics. Assay sensitivity, specificity and reproducibility are the minimal evaluations that have to be performed (Avet-Loiseau, 2016; Kim et al., 2017; Rehm et al., 2013). Sensitivity of the FLT3-ITD NGS assays has been published in several studies but detailed analysis of reproducibility, especially at low FLT3-ITD mutant rates has not been presented, yet, partly due to the absence of proper quality control materials. We created a DNA pool of AML patients' samples containing several different ITDs - with different sizes and rates - and show that such a QC material can be used properly for analytical evaluation of FLT3-ITD amplicon-based NGS assays.

2. Materials and Methods

2.1 DNA pool preparation from samples of patients with AML

Genomic DNA was isolated from peripheral blood samples of patients with AML using the QIAmp DNA Blood Mini kit (Qiagen, Valencia, CA). As part of their routine diagnostic evaluation the FLT3-ITD determination was performed as described before (Buban et al., 2011). Six FLT3-ITD positive DNA samples were pooled based on the size and rate of their FLT3-ITDs as described in Supplementary Table 1. The final mix - named as QC sample - contained 8 different FLT3-ITDs ranging in size between 9 and 71 bp and in ITD/total allele rate between 0.15% and 13.6%.

2.2 Amplification of the juxtamembrane region of FLT3 involved in ITD development

The region of exon 14, intron 14 and exon 15 of the FLT3 gene was amplified using the primers and conditioned published by Buban and colleagues (Buban et al., 2011). The primers contained additional adapter sequences for emulsion PCR and sample specific barcode sequences (multiplex identifiers).

2.3 Deep next generation sequencing of the FLT3 amplicons

DNA libraries were prepared using the Roche Rapid Library kit according to the Rapid Library Preparation Method Manual. Pyrosequencing was carried out on a GS Junior (Roche 454 Life Sciences, Branford, CT, US) using the GSJR titanium sequencing kit according to the Roche Sequencing Method Manual. Amplicons were sequenced from both forward and reverse ends.

In order to correctly identify each FLT3-ITD variant, even the rare ones, the data obtained from each run was exported in tabular delimited files (csv file type), where each line contained a sequence corresponding to a single read. In the next step each individual read was compared to the sequence of the wild type and the known FLT3-ITD variants and a Levenshtein distance score was computed (Levenshtein, 1966; Navarro, 2001). A difference of 5 or less nucleotide was treated as an amplification error. Reads with no difference or less than 5 nucleotide changes compared to wild type or one of the FLT3-ITD variants were counted as a member of the respective variant group. In all other cases, manual sequence analysis was performed to identify the respective FLT3-ITD mutation (variant).

2.4 Statistical analysis

FLT3-ITD/total allele rates of variants detected in duplicate samples of the same days (intra-assay variance) were compared by linear regression analysis, while association between the

number of ITD variant types and the number of total NGS reads were determined by hyperbolic curve fitting using the GraphPad Prism software (version 7.04). Significant difference was accepted at $p < 0.05$.

3. Results and Discussion

3.1 Sensitivity of NGS based FLT3-ITD quantitation

In total 7 separate runs were performed on 7 different days and the average read number in one sample of a run was 14,305 (range: 9,395 - 33,818). Altogether 19 FLT3-ITD variants were identified (Table 2 and Supplementary Table 2), which was higher than the number of ITDs found by the routine PCR + capillary electrophoresis method, proving higher sensitivity of the NGS based FLT3-ITD assay. While our PCR+CE assay has a maximal sensitivity of 0.3% (Buban et al., 2011), the theoretical sensitivity of the NGS FLT3-ITD assay used by us is 0.003% (1 positive read per the total maximal 33,818 reads). Several studies reported variable sensitivity for NGS based quantitation of FLT3-ITD allele rate: 0.01% (Thol et al., 2012), 1.0% (Spencer et al., 2013), 1.5% (Luthra et al., 2014), 0.125% (Zuffa et al., 2015), 0.005% (Bibault et al., 2015), 0.5% (Schranz et al., 2018) and 0.005% (Levis et al., 2018) has been reported. Even higher sensitivities – upto 0.0001% - were described in other NGS applications, (Avet-Loiseau, 2016; Takamatsu, 2017). It is very likely that the maximal achieved read count in a sample is an important determinant of the sensitivity of an NGS assay. In order to asses this theory our QC sample was tested in a series of runs where - using barcode identifiers, and altered mixing rates of the purified amplicons - different total read numbers (from n=858, to n=33,818) were created in each sample (Fig. 1). The smallest number of FLT3-ITD variant types detected was 7, the maximum was 19, beside the wild type. There was a direct correlation between read numbers and variant types detected in a single run. Below 4000 reads the number of ITD types detected varied between 7 and 12,

while between 4000 and 10000 reads this range was 12-17. Above 10,000 reads the number of ITD variants changed much less (15-19). These data show that, indeed, there is a significant correlation between the sensitivity of the NGS assay and the read count, therefore, increasing the total number of reads per sample can enhance the ability of these assays to detect minimal residual disease.

3.2 Reproducibility of NGS based FLT3-ITD quantitation

Another important issue in NGS assay validation is assessment of reproducibility. Until now, only sporadic studies provided information about the performance of FLT3-ITD NGS tests. Thol characterized the reproducibility of their FLT3-ITD NGS assay in two independent runs with acceptable results, testing samples with ITD allele frequency from 22% to 66% (Thol et al., 2012). Zuffa reported an intra- and inter-assay CV%=3% measuring one FLT3-ITD positive sample with 50% ITD rate, in two independent runs, in triplicates (Zuffa et al., 2015). In a recent report Schranz and her colleagues provided data about their FLT3-ITD NGS assay. The inter-assay reproducibility was CV%=0.17% testing a sample with 49.2% ITD rate in 4 independent runs (Schranz et al., 2018). These three studies tested only one or three ITD variants present in high rates in the control samples and the number of tested replicates were also low. Since these NGS tests need to be able to quantify different and low levels of ITD variants when we use them as MRD assays, the reproducibility of them has to be evaluated at different and low levels of ITD frequencies, as well. Our QC sample can also be used for this purpose.

The intra-assay variability tested by the QC sample containing 19 different ITD variants at different levels is presented in Fig. 2. Data from three separate days are shown together. On each days the QC sample was tested in barcoded duplicates, the number of total reads per sample was always above 10,000. The coefficient of correlation for the three days

combined was R=0.954. When analyzed separately, the correlation coefficient was R=0.904, R=0.983 and R=0.985, respectively (data not shown). Nonetheless, on Fig. 2 the data points show somewhat higher variability around an ITD rate of 0.1% and it is more expressed at around 0.01%.

Inter-assay variability was assessed in seven independent days using our QC sample, having at least 10,000 individual reads per sample. The mean, SD and CV% values were calculated for each identified ITD variants presented in Table 2. Our results show - similarly to the former 3 mentioned studies - that in the presence of high ITD allele frequencies (in our case 78% is the wild type allele) the reproducibility of the FLT3-ITD NGS assay is really high. An analytically acceptable CV% (<25%) is achievable if the ITD rate is above 0.1%. Although ITDs can be detected even below this cut-off value, their quantification has low reproducibility (Table 2).

Conclusions

In order to evaluate the analytical properties of an NGS-based FLT3-ITD detection assay a QC sample was prepared from DNA of AML patients. While the routine diagnostic PCR+CE assay revealed 8 different FLT3-ITD variants in the QC mix, NGS could identify 19 different FLT3-ITD sequences with different sizes and rates. The higher the total read count was in a certain sample of the NGS run, the more ITD variant types could be detected. The maximal sensitivity of FLT3-ITD detection by NGS technique was as low as 0.007% FLT3-ITD/total allele rate, however, below 0.1% rate, the reproducibility of the quantitation was poor (CV% was higher than 25%). Our data indicate that DNA pools of FLT3-ITD positive DNA samples can be used efficiently for the analytical validation of FLT3-ITD detecting NGS assays.

Ethics approval and consent to participate: The research performed has been complied with all of the relevant national regulations, institutional policies and in accordance to the tenets of the Helsinki Declaration, and has been approved by the Ethical and Science Committee of the University of Debrecen.

Consent for publication: Informed consent was obtained from all individuals included in the study.

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest: The funding organizations played no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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Authors' contributions: ZM participated in the genetic experiments, performed FLT3-ITD variant identification and assisted in writing the manuscript. TD participated in FLT3-ITD variant identification, statistical analyses and manuscript writing. RF performed PCR+CE analysis and performed partly the NGS assay. LM participated in the NGS assay. AS participated in PCR+CE and statistical analysis and manuscript writing. MP and PASZ conceived the experiments and wrote the manuscript. All authors have accepted responsibility for the entire content of the submitted manuscript and approved submission.

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Legends for the Figures

Fig. 1. Association of total read count and number of FLT3-ITD variants. The relationship between the number of FLT3-ITD variants found in a single run (y axis) and the total number of reads in that run (x axis) is shown. The best curve that could be fit to these points was provided by a hyperbolic equation: $y = (19.69*x) / (1170+x)$

Fig. 2. Correlation of FLT3-ITD rates between QC sample duplicates run on the same day. The QC sample was amplified twice - by two primer pairs with different multiplex identifiers (sample 1 and sample 2) - and was evaluated in the same run at 3 different days. The FLT3-ITD/total allele rate % is presented on a logarithm scale for the wild type and the 19 FLT3-ITD variants obtained in sample 1 and sample 2. Results of day 1, 2 and 3 are presented by rhomboid, square and rectangle symbols. If a variant was not identified in a sample it is presented on the Figure as N.D. (not detected), and these points were omitted from the calculation of linear regression.

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Table 1

Comparison of methods used for detection of FLT3-ITD

	TD-PCR	PCR + CE	RQ-PCR	Sanger sequencing	NGS
ITD size detection	YES*	YES	NO	YES	YES
ITD rate quantitation	YES	YES	YES	NO	YES
ITD sequence identification	NO	NO	NO	YES	YES
Multiple ITD detection	LIMITED**	YES	NO	NO	YES
Patient specific primers/probes are required	NO	NO	YES	NO	NO
Sensitivity	0.001%	0.3%	0.001%***	10-20%	0.0001%
Turn-around time	1 DAY	1 DAY	1 DAY	1-2 DAYS	3-5 DAYS

TD-PCR: tandem duplication PCR; CE: capillary electrophoresis; RQ-PCR: real-time quantitative PCR; NGS: Next generation sequencing; *FLT3-ITDs shorter than 40 bp are not detected; **Only if the two FLT3-ITD mutations are far from each other; ***Depends on the primers used.

Table 2

Inter-assay reproducibility of the rate of FLT3-ITD variants.

FLT3-ITD	MEAN	SD	CV	Times detected
wild type	78.239%	2.746%	3.5%	7
c.1741_1776dup36	12.442%	1.690%	13.6%	7
c.1757_1795dup39	3.488%	0.474%	13.6%	7
c.1757_1783dup27	1.912%	0.346%	18.1%	7
c.1752_1826dup75	1.343%	0.224%	16.7%	7
c.1788_1789insGGGGTCCCT	1.019%	0.112%	11.0%	7
c.1789_1837+2dup51	0.830%	0.105%	12.6%	7
c.1768_1833dup66	0.253%	0.039%	15.3%	7
c.1727_51delinsTCAAATGGGAGTTTCCAAGAGAAAA	0.118%	0.028%	23.3%	7
c.1777_1818dup42	0.108%	0.028%	25.4%	7
c.1804_1805insCGGCCTACGTTGATTTCAGAGAATATGAATATGATCTCA	0.066%	0.079%	121.0%	5
c.1741_1776dup36 + c.1789_1837+2dup51	0.028%	0.011%	39.2%	7
c.1741_1803dup63	0.021%	0.006%	28.2%	7
c.1741_1776dup36 + c.1752_1826dup75	0.016%	0.016%	98.0%	6
c.1773_1793dup21	0.013%	0.008%	60.0%	7
c.1741_1776dup36 + c.1788_1789insGGGGTCCCT	0.011%	0.006%	54.2%	6
c.1741_1776[3]	0.010%	0.005%	50.0%	7
c.1745_1819dup75	0.009%	0.004%	41.1%	7
c.1747_1794dup48	0.007%	0.005%	66.9%	6
c.1741_1776dup36 + c.1757_1795dup39	0.007%	0.005%	82.1%	5

The FLT3-ITD/total allele rate (%) was calculated in each experimental day (n=7) for each variant. The mean, standard deviation (SD) and the coefficient of variation (CV%) is shown based on the data from the 7 separate days. The last column shows the number of days when the certain variants could be detected.

Figure 1

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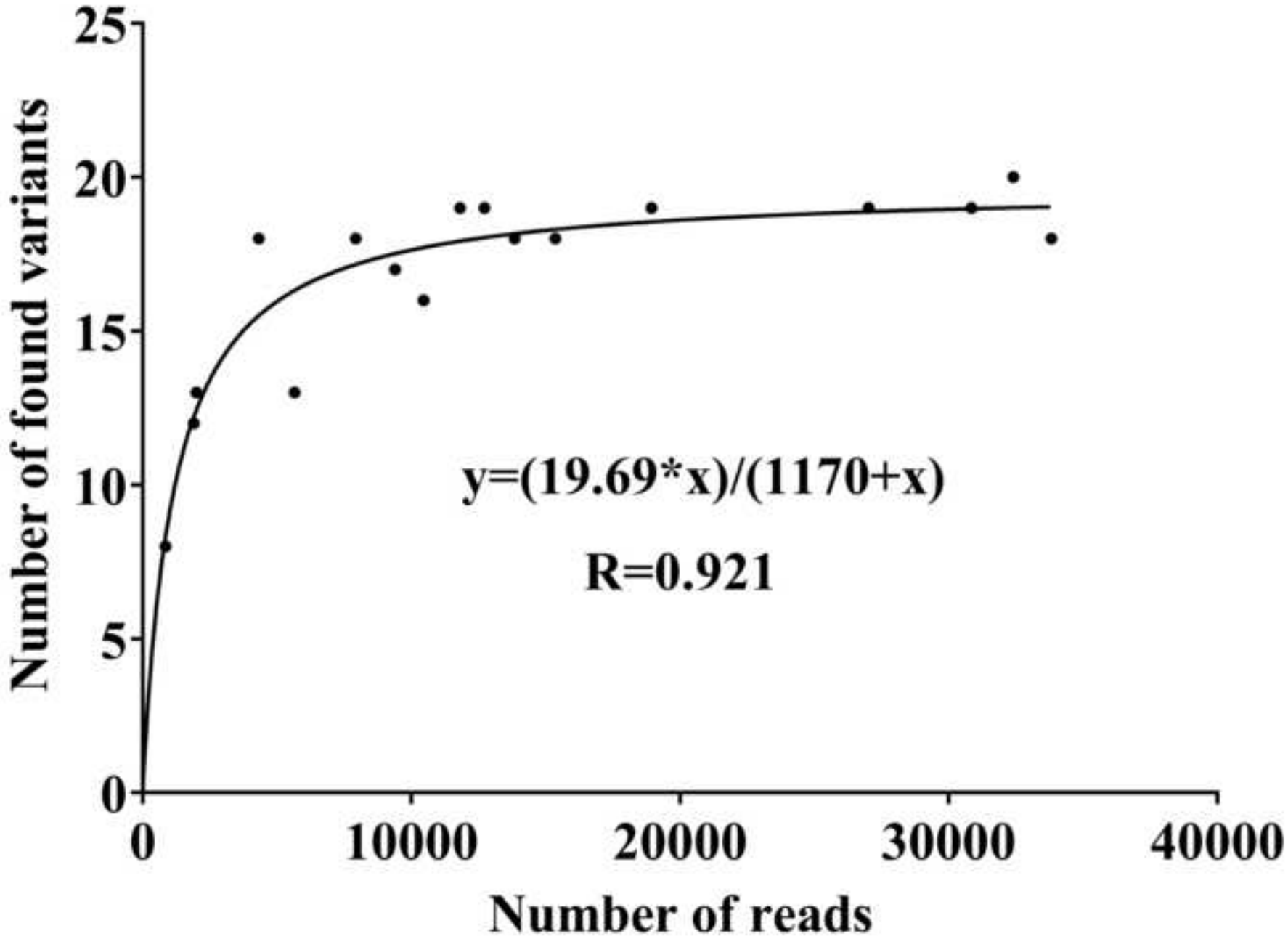

Figure 2

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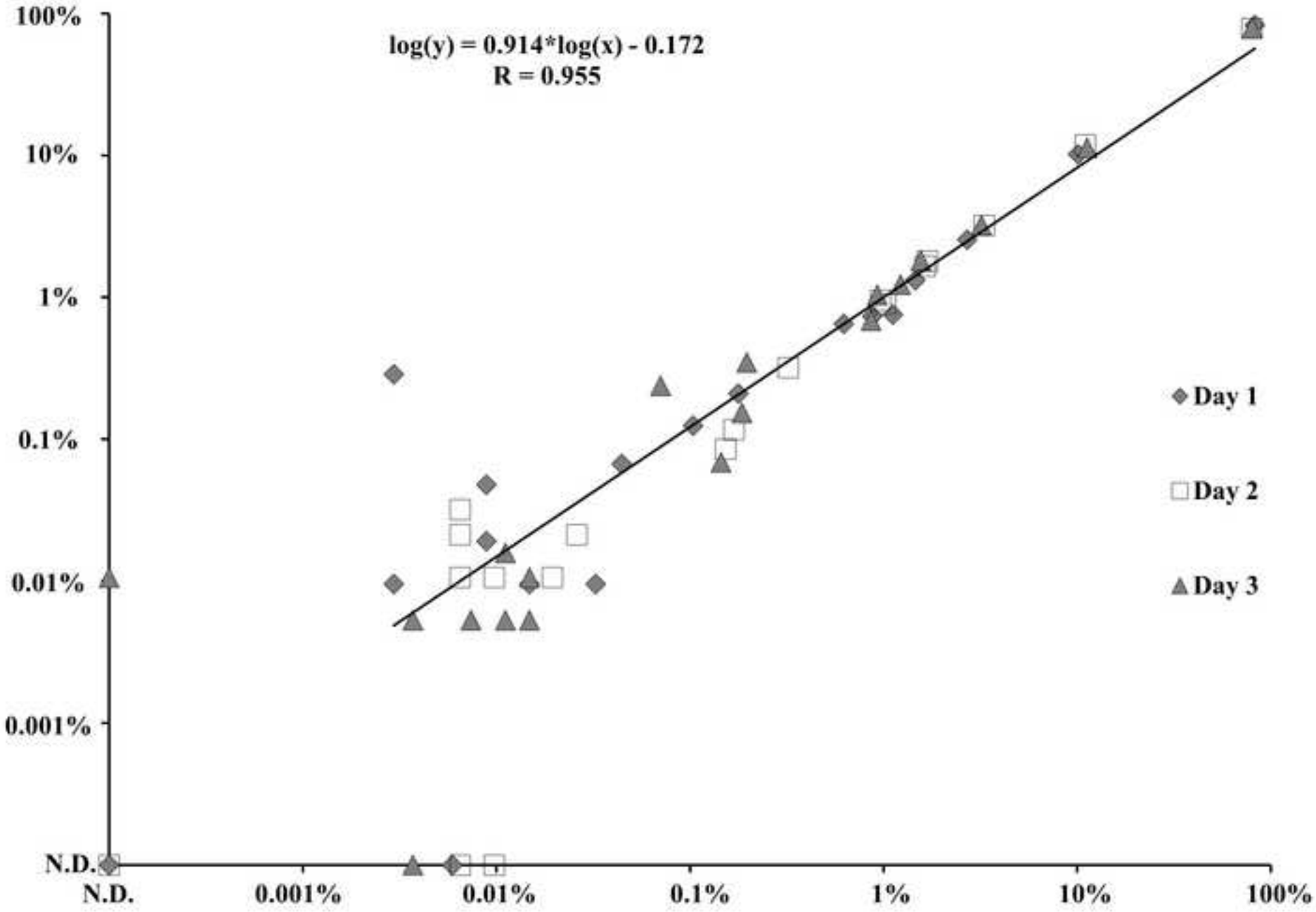

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Internal tandem duplication (ITD) in the fms-like tyrosine kinase 3 (*FLT3*) gene is one of the most frequent genetic alteration in acute myeloid leukemia (AML), and it is associated with worse clinical outcome. Not only the presence but also the size, localization and the rate of this variant or the presence of multiple ITDs has prognostic information. The traditional PCR based diagnostic methods cannot provide information about all of these parameters in one assay, however the application of next generation sequencing (NGS) technique can be a reliable solution for this diagnostic problem. In order to evaluate the analytical properties of an NGS-based FLT3-ITD detection assay a QC sample was prepared from DNA of AML patients containing 19 different FLT3-ITD variants identified by NGS. The higher the total read count was in a certain sample of the NGS run, the more ITD variant types could be detected. The maximal sensitivity of FLT3-ITD detection by NGS technique was as low as 0.007% FLT3-ITD/total allele rate, however, below 0.1% rate, the reproducibility of the quantitation was poor (CV>25%). DNA pools with several FLT3-ITDs can be used efficiently for analytical evaluation of NGS-based FLT3-ITD quantitation testing several different ITD sequences and rates, simultaneously.